

# **Standard Procedures for Aquatic Animal Health Inspections**

**Developed and Edited by:**

**United States Fish and Wildlife Service  
And  
American Fisheries Society – Fish Health Section**

# **Chapter 3**

## **Bacteriology**

### **3.1 Introduction**

The following chapter describes inspection procedures for bacterial pathogens of fish that may be required for a fish health inspection. The target bacterial species include *Aeromonas salmonicida*, *Yersinia ruckeri*, *Edwardsiella ictaluri* and *Renibacterium salmoninarum*. Chapter 2 describes procedures for proper sampling of fish tissues to ensure detection of any of these pathogens during a fish health inspection.

Presumptive identifications of *A. salmonicida* (subspecies *salmonicida* and *achromogenes*) (3.2), *E. ictaluri* (3.4), and *Y. ruckeri* (3.3) are based on Gram staining properties, and characteristic biochemical reactions. Confirmatory identification consists of fluorescent antibody testing using fluorescein-conjugated, species-specific antibody (3.7.E). Known isolates of *A. salmonicida*, *E. ictaluri* and *Y. ruckeri* are purchased from ATCC and are used as positive controls. Single, unknown isolates may be used to test for all three of these organisms.

The presumptive identification of the Gram-positive bacterium *R. salmoninarum* (3.5) is based upon serological methods. For purposes of initial screening and detection of the pathogen, the direct fluorescent antibody technique (FAT) on kidney smears and ovarian fluid samples employed (3.7.E). Documentation exists which indicates the possibility for false positive results caused by bacterial organisms which cross react with antibodies prepared against *R. salmoninarum* (Austin, 1985; Bullock, 1980; Brown, et al, 1995). For this reason, it is important to follow steps described below to confirm that a positive FAT result is due to the presence of this pathogen. **Exception:** Anadromous salmonids regularly monitored for *R. salmoninarum* with ELISA or quantitative PCR techniques may be considered positive without additional testing by FAT.

Any FAT results which appear positive for *R. salmoninarum* should be confirmed by either culture of kidney tissue on selective kidney disease medium (SKDM-2) (3.5.B.1), or by testing the positive tissues with the polymerase chain reaction (PCR) technique (3.5.B.2).

*DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.*

### **3.2 *Aeromonas salmonicida* (Furunculosis)**

*Aeromonas salmonicida* may be difficult to detect based on a variety of environmental, physiological and host factors. At water temperatures between 14-24°C, fish can develop clinical disease within 4-12 days after bacterial exposure in the water supply. At temperatures below 13°C, chronic and latent infections are more likely to develop. This pathogen has been associated with disease in a variety of salmonid and non-salmonid species. It is generally accepted that any freshwater fish can carry the bacteria with or without showing signs of disease (Thoesen, 1994, Bullock, et al. 1983). The typical form of the bacterium (subspecies *salmonicida*) produces a soluble brown pigment on tryptic soy agar after two to three days of growth at 20°C. The less common and atypical strain of this pathogen (subspecies *achromogenes*) does not produce pigmentation under these incubation conditions. Both strains, however, have been associated with disease in a variety of fish species (Paterson et al. 1980).

#### **A. Summary of Screening Test**

##### **Bacterial Culture and Biochemical Analysis**

1. Aseptically inoculate samples into TSA tubes or onto plates as described in Chapter 2
2. Incubate for 24-48 hours at 20 - 24°C (room temperature). If no growth occurs at either 24 or 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are reported as negative for *A. salmonicida*.**
3. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single, isolated colony to subculture onto fresh TSA plates. If colonies are not well isolated on the original media, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.
4. Incubate at 20-24°C for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.
5. Using a sterile needle or small loop, pick individual distinct bacterial colonies representing each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each isolated colony and record all colony characteristics on the data sheet.
6. Begin initial identification of pure strain bacterial cultures (3.A1):
  - a. Colony Pigmentation: Typical strains of *A. salmonicida* have brown diffusible pigment after 2-3 days of incubation. Some strains may not be pigmented (subsp. *achromogenes*).
  - b. Gram Determination (3.7.A): *A. salmonicida* is Gram Negative. Gram Positive isolates may be reported as negative for *A. salmonicida*.
  - c. Presence of Cytochrome Oxidase (CO) (3.7.B): Most are CO positive. Rarely, CO negative strains of *A. salmonicida* subsp. *salmonicida* have been encountered (Chapman, et al. 1991).

- d. Motility (3.7.C): *A. salmonicida* is non-motile. Motile isolates may be reported as negative for *A. salmonicida*.
- 7. Perform biochemical testing on each isolate (3.A1):
  - a. Tube Method (3.7.D.1)
    - i. Glucose fermentation (3.7.D.1.a) using OF basal medium containing glucose will produce an Oxidative/fermentative (O/F) or a Fermentative (F) result with Gas (most strains produce gas, but some may be weak or variable in this production). **Any isolate with a result other than this may be reported as negative for *A. salmonicida*.**
    - ii. Gelatinase (3.7.D.1.c) and Indole (3.7.D.1.d) are considered together with the pigmentation of the isolate for interpretation of the results.
      - 1. Brown diffusible pigmented isolates that are Gelatinase positive and Indole negative are **PRESUMPTIVELY positive *A. salmonicida* sub-species *salmonicida*.**
      - 2. Non-pigmented isolates that are Gelatinase negative and Indole positive are **PRESUMPTIVELY positive *A. salmonicida* sub-species *achromogens*.**
      - 3. **Isolates yielding any other combination of these results may be reported as negative for *A. salmonicida*.**
  - b. Commercial Identification Systems (3.7.D.2)
    - i. Biolog (3.7.D.2.b)
    - ii. API: If isolates are tested with the commercial system API described in Section 3.7.D.2.a, it is recommended that the Reference profiles be consulted in 3.A2.
- 8. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.
- 9. Positive control isolates of *Aeromonas salmonicida* can be obtained from the American Type Culture collection (ATCC). The internet location for ATCC is <http://www.atcc.org>. Below are suggested isolates to use for positive control cultures:
  - a. *A.salmonicida* subspecies *salmonicida* – ATCC # 14174
  - b.*A.salmonicida* subspecies *achromogenes* – ATCC # 10801
  - c. A **cytochrome oxidase negative** isolate is also available – ATCC # 49385

## B. Confirmatory Test

**Fluorescent Antibody Test (FAT)** (3.7.E) is performed on at least one representative isolate from each lot inspected and found positive during screening. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control. A list of sources from which antibodies may be obtained is provided in 3.7.E.

### **3.3 *Yersinia ruckeri* (Enteric Redmouth Disease, ERM)**

*Yersinia ruckeri* affects fingerlings of potentially all salmonids. In salmonids, mortality increases dramatically following periods of stress due to environmental conditions and handling. Survivors of enteric redmouth disease outbreaks become carriers, after which the bacteria are shed from the intestinal tract in large numbers during a regular 36 to 40 day cycle. Severity of infection decreases at water temperatures below 10°C. *Y. ruckeri* has been isolated from the kidney of some non-salmonids as well. It is generally accepted that, although not ubiquitous in the environment, any freshwater fish can carry the bacteria with or without exhibiting signs of disease (Thoesen, 1994; Bullock, 1984).

#### **A. Summary of Screening Test**

##### **Bacterial Culture and Biochemical Analysis**

1. Aseptically inoculate samples onto tubes or plates as described in Chapter 2
2. Incubate for 24-48 hours at 20 - 24°C, (room temperature). If no growth occurs at either 24 or 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are discarded and reported as negative for *Y. ruckeri*.**
3. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single isolated colony to subculture onto fresh TSA or BHIA plates. If colonies are not well isolated on the original media, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.
4. Incubate at 20 - 24°C for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.
5. Using a sterile needle or small loop, pick individual distinct bacterial colonies to represent each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.
6. Begin initial identification of pure strain bacterial cultures (3.A1):
  - a. Gram Determination (3.7.A): *Y. ruckeri* is Gram Negative. **Gram Positive isolates may be reported as negative for *Y. ruckeri*.**
  - b. Presence of Cytochrome Oxidase (CO) (3.7.B): *Y. ruckeri* is CO negative. **CO positive isolates may be reported negative for *Y. ruckeri*.**
  - c. Motility (3.7.C): *Y. ruckeri* is motile. **Non-Motile isolates may be reported as negative for *Y. ruckeri*.**
7. Perform biochemical testing on each isolate (3.A1):
  - a. Tube Method (3.7.D.1)
    - i. Triple Sugar Iron (TSI) (3.7.D.1.b): *Y. ruckeri* will yield an Alkaline over Acid (K/A) or Alkaline over Acid with Gas (K/AG) result. **Any**

**isolate with a result other than this may be reported as negative for *Y. ruckeri*.**

- ii. Carbohydrate Utilization (3.7.D.1.e):
  - 1. Arabinose cannot be utilized (fermented) by *Y. ruckeri*.
  - 2. Rhamnose cannot be utilized (fermented) by *Y. ruckeri*.
  - 3. Sucrose cannot be utilized (fermented) by *Y. ruckeri*.
  - 4. Salicin cannot be utilized (fermented) by *Y. ruckeri*.
  - 5. **Isolates yielding positive results for any of these tests may be reported as negative for *Y. ruckeri*.**
- iii. Malonate Test (3.7.D.1.g): **Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.**
- iv. Indole Test (3.7.D.1.d): **Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.**
- v. Esculin Test (3.7.D.1.h): **Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.**
- vi. Decarboxylase Test (Lysine) (3.7.D.1.f): **Isolates yielding negative results for this test may be reported as negative for *Y. ruckeri*.**
- vii. Sorbitol (3.7.D.1.e):
  - 1. Isolates that satisfy all previous conditions in this section are **PRESUMPTIVELY positive** for *Y. ruckeri*.
  - 2. Sorbitol is used to differentiate between Type I and Type II *Y. ruckeri*.
    - a. Isolates yielding negative results for this test are **PRESUMPTIVELY positive** for *Y. ruckeri* Type I.
    - b. Isolates yielding positive results for this test are **PRESUMPTIVELY positive** for *Y. ruckeri* Type II.
- b. Commercial Identification Systems (3.7.D.2)
  - i. Biolog (3.7.D.2.b)
  - ii. API: If isolates are tested with the commercial system API described in Section 3.7.D.2.a, it is recommended that the Reference profiles be consulted in 3.A2.



8. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.
9. Positive control isolates of *Yersinia ruckeri* can be obtained from the American Type Culture collection (ATCC). The internet location for ATCC is <http://www.atcc.org>. Below are suggested isolates to use for positive control cultures:
  - a. *Yersinia ruckeri* serovar Type I – ATCC # 29473
  - b. *Y. ruckeri* Type II – ATCC # 29908

## **B. Confirmatory Test**

**Fluorescent Antibody Test (FAT)** (3.7.E) is performed on at least one representative isolate from each lot inspected and found positive during screening.

- 1 A separate antibody must be utilized for confirmation of either Type I or II strains.
- 2 Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control.
- 3 A list of sources from which antibodies may be obtained is provided in 3.7.E.

### **3.4 *Edwardsiella ictaluri* (Enteric Septicemia of Catfish, ESC)**

The primary host species of this pathogen include all species of catfish, tilapia, as well as other warm water species. Both fingerlings and adults can be affected by the disease. In catfish, the bacterium is transmitted through the olfactory system to the brain, where the typical “hole in the head” lesions can be observed during an *E. ictaluri* epizootic. Disease typically occurs at water temperatures between 20°C and 30°C. Experimental infections have been established in salmonids. It is generally accepted that any warm water species of fish can carry this pathogen with or without exhibiting signs of disease (Austin & Austin, 1987; Thoesen, 1994, Bullock & Herman, 1985; Hawke et al, 1981).

#### **A. Summary of Screening Test**

##### **Bacterial Culture and Biochemical Analysis**

- 1 Aseptically inoculate samples onto tubes or plates as described in Chapter 2.
- 2 Incubate for 24-48 hours at 28 - 30°C. Alternatively this organism can be grown at 20 - 24°C (Plumb et al, 1989). If no growth occurs at 24 and 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are discarded and reported as negative for *E. ictaluri*.**
- 3 When primary culture occurs on tubes or plates use a sterile loop or needle to select a single colony to subculture onto fresh TSA or BHIA plates. If colonies are not well isolated, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.
- 4 Incubate at temperature used above for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.
- 5 Using a sterile needle or small loop, pick individual distinct bacterial colonies to represent each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.
- 6 Begin initial identification of pure strain bacterial cultures (3.A1):
  - a. Gram Determination (3.7.A): *E. ictaluri* is Gram Negative. **Gram Positive isolates may be reported as negative for *E. ictaluri*.**
  - b. Presence of Cytochrome Oxidase (CO) (3.7.B): *E. ictaluri* is CO negative. **CO positive isolates may be reported negative for *E. ictaluri*.**
- 7 Perform biochemical testing on each isolate (3.A1):
  - a. Tube Method (3.7.D.1)
    - i. Triple Sugar Iron (TSI) (3.7.D.1.b): *E. ictaluri* will yield an Alkaline over Acid (K/A) or Alkaline over Acid with Gas (K/AG) result. **Any**

**isolate with a result other than this may be reported as negative for *E. ictaluri*.**

- ii. Carbohydrate Utilization (3.7.D.1.e):
  - 1. The following carbohydrates cannot be utilized (fermented) by *E. ictaluri*:
    - Arabinose
    - Rhamnose
    - Sucrose
    - Salicin

**2. Isolates yielding positive results for any of these tests may be reported as negative for *E. ictaluri*.**

- iii. Malonate Test (3.7.D.1.g): **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.**

- iv. Indole Test (3.7.D.1.d): **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.**

- v. Esculin Test (3.7.D.1.h): **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.**

- vi. Decarboxylase Test (Lysine) (3.7.D.1.f): **Isolates yielding negative results for this test may be reported as negative for *E. ictaluri*.**

- vii. Manitol Utilization Test (3.7.D.1.e):
  - 1. **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.**
  - 2. Isolates yielding negative results for this test and that satisfy all previous conditions in this section are **PRESUMPTIVELY positive** for *E. ictaluri*

b. Commercial Identification Systems (3.7.D.2)

- i. Biolog (3.7.D.2.b)
- ii. API: If isolates are tested with the commercial system API described in Section 3.7.D.2.a, it is recommended that the Reference profiles be consulted in 3.A2.

10 When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

11. Positive control isolates of *Edwardsiella ictaluri* can be obtained from the American Type Culture collection (ATCC). The Internet location for ATCC is <http://www.atcc.org>.

## **B. Summary of Confirmatory Test**

**Fluorescent Antibody Test (FAT)** (3.7.E) is performed on at least one representative isolate from each inspection. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control. A list of sources from which antibodies may be obtained is provided in 3.7.E.

### **3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD)**

*Renibacterium salmoninarum* infections can occur at any life stage in salmonid populations. Clinical signs of disease are uncommon in fish less than 6 months of age. Mortality has been reported at water temperatures between 4°C and 20.5°C, with the disease progressing rapidly between 15°C to 20.5°C. Acute and sub-acute forms of disease are less common than the more typical chronic form of disease, characterized internally by a large edematous kidney that appears gray and corrugated (Thoesen, 1994; Bullock & Herman, 1988; Austin, 1987).

#### **A. Summary of Screening Test**

##### **Fluorescent Antibody Test (FAT) (3.7.E)**

Collect tissues as described in Chapter 2 and prepare FAT slides (**Exception:** Anadromous salmonids regularly monitored for *R. salmoninarum* with ELISA, quantitative PCR, or MFAT techniques may be considered positive without additional testing by FAT).

1. Collect tissues as described in Chapter 2 and prepare FAT slides:
  - a. Kidney – prepare kidney smear on a non-coated or acetone-cleaned glass slide
    - i. Place a piece of posterior kidney or homogenized preparation on the slide.
    - ii. Create a thin smear on the surface of the glass slide.
  - b. Ovarian Fluid pellet smear:
    - i. After pooled ovarian fluid samples are processed and the appropriate amount of supernatant removed for virology assays (see 4.4.C), the pellet is re-suspended in the remaining ovarian fluid by thorough vortexing or repeat pipetting.
    - ii. Transfer two 1.5 mL aliquots from each original pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL microcentrifuge tubes (see **Note**). Freeze the remainder of the sample at -20°C for PCR confirmation.
    - iii. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes (see **Note**).
    - iv. The pellet is carefully removed with a small amount of supernatant using a sterile pipette and a thin smear is prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

**Note:** Elliot and McKibben (1997) document the importance of using a minimum of 10,000 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL of ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e. 2.5 mLs from a 5-fish-

pool). Most 15 mL centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal force (rcf) as high as 10,000 X g. It is, therefore, necessary to aliquot the appropriate amount of fluid into microcentrifuge tubes suitable for the required rcf.

2. After the tissue smear is heat fixed or air dried, slides are fixed in acetone for 5 minutes.
3. Stain slides with FITC-conjugated *R.salmoninarum* antisera as described in 3.7.E.2.
4. Examine at least 50 fields using oil immersion at 1000X magnification to detect the 1.0 X 0.5 micron bacterial cells, which should appear stained as an “apple green” fluorescence.
  - a. **Smears which do not show any fluorescent bacterial cells may be discarded and reported as negative for *R. salmoninarum*.**
  - b. Any smears, which have “apple green” fluorescent, diplo-bacilli bacterial cells present measuring approximately 1.0 X 0.5 microns, shall be considered **PRESUMPTIVELY positive** for *R. salmoninarum*.
5. It is preferable to use tissue infected with *R.salmoninarum* for a positive FAT control. Positive control culture isolates of *Renibacterium salmoninarum*, however, can be obtained from the American Type Culture collection (ATCC). The internet location for ATCC is <http://www.atcc.org>. Suppliers of commercially prepared antibodies for FAT may also provide positive control materials for use in this assay (see 3.7.E source list).

## **B. Confirmatory Tests**

- 1 **Bacterial Culture** (Austin et al,1983)
  - a. At the time samples are collected during the inspection (2.2.E.2), aseptically inoculate samples of tissues onto plates containing selective kidney disease media (SKDM-2) (3.6.A.3).
  - b. Incubate for 2-3 weeks at 15°C in a humid chamber to prevent dehydration of media.
  - c. At 2-3 weeks, observe plates for growth of pinpoint bacterial colonies.
    - i. If *R. salmoninarum* is presumptively identified in FAT, corresponding samples inoculated onto SKDM-2 should be examined weekly.
    - ii. If no growth, continue to incubate plates for up to 6 weeks, and examine them several times per week for growth.

- iii. **If no growth after 6 weeks samples may be discarded and reported as negative for *R. salmoninarum*.**
- iv. If growth of small (2 mm diam.), smooth, white round colonies is observed, obtain inoculum from colony and confirm identification using FAT or PCR.
  - 1. **If FAT or PCR results on culture are positive sample is reported as positive for *R. salmoninarum*.**
  - 2. **If FAT or PCR results on culture are negative sample is reported as negative for *R. salmoninarum***

**Note:** The slow growth of this organism makes phenotypic characterization of suspect isolates difficult and time consuming. The inspector may consider pursuing phenotypic characterization if the detection of *R. salmoninarum* by these techniques continues to be questionable (consult Austin and Austin, 1993).

## **2. Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA**

The Polymerase Chain Reaction technique employs oligonucleotide primers to amplify segments of the gene that codes for the 57 kDa protein of *R. salmoninarum* (Chase and Pascho, 1998). DNA is extracted from fish tissues and amplified initially with forward and reverse primers. The amplified DNA product is then re-amplified using a “nested PCR” technique. The DNA products from both amplifications are then visualized by agarose gel electrophoresis. The following procedures have been adapted from those of Chase and Pascho (1998), and have been reviewed and approved by the authors.

- a. Extraction of DNA from Kidney and Ovarian Tissues (**NOTE:** The following procedure employs an extraction kit available from Qiagen, Inc. (<http://www.qiagen.com>). DNA Extraction kits of similar efficacy are available from many other sources, and can be utilized as alternatives for extraction of DNA in this protocol. These kits utilize “spin columns” for binding and elution of DNA from tissue lysates. Most do not require the use of highly toxic reagents and reduce the chance of contamination during extraction).
  - i. Procedures:
    - 1. Transfer 25-50 mg of kidney tissue, or 50µl ovarian fluid, into a 1.5 ml Micro centrifuge tube. Tissue can be fresh or previously frozen.
    - 2. Add 180µl of lysozyme lysis buffer (3.6.G.1). Incubate at 37°C for 1 hour, vortexing occasionally.

3. Add 25µl of Proteinase K stock solution and 200 µl of buffer AL lysis buffer (provided by extraction kit manufacturer), then mix by vortexing and incubate at 70°C for 30 minutes. Vortex occasionally. Tissues should be well lysed by the end of 30 minutes.
4. Incubate at 95°C for another 10 minutes. Vortex occasionally
5. Add 210µl of ethanol, mix thoroughly on vortex
6. Place a spin column in a 2ml collection tube. Place sample mixture over the filter in the spin column, being careful not to moisten the rim of the column. Close the cap and centrifuge at 6000 x g for 1 minute at room temperature.
7. Place the spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
8. Carefully open spin column and add 500µl Buffer AW1 (wash buffer provided by kit manufacturer). Centrifuge again as described above.
9. Repeat steps 7 and 8, using 500µl Buffer AW2. Centrifuge at full speed for 3 minutes to dry the membrane.
10. Place spin column in clean 1.5 ml Micro centrifuge tube and add 200µl of Buffer AE (elution buffer provided by kit manufacturer) for elution of DNA (TE buffer pH 8.0 or water can also be used). Incubate for 5 minutes at room temperature. Centrifuge at 6000 x g for 1 minute.
11. Repeat step 10 so that the total volume of DNA is 400µl. Discard spin column and store DNA solution at -20-70°C until use.
12. Quantify the amount of DNA extracted with a spectrophotometer (Chapter 6).
  - a. 25-50 mg of fish kidney tissue should produce between 100 and 300 ng DNA per µl using this procedure. A greater concentration of DNA should be diluted with elution buffer before performing PCR.
  - b. 50 µl ovarian fluid produces a much lower amount of DNA per µl using this procedure. Dilute the template if DNA exceeds 300 ng/µl.

b. Initial Amplification of *R. salmoninarum* DNA



- i. General QA/QC Considerations must be considered before performing PCR (See Chapter 6 for more specific QA/QC considerations for PCR).
- ii. Procedures for initial round:
  1. Using Worksheet 3.A3.A “DNA Samples”, Record appropriate data for each sample to be tested by PCR.
  2. Using Worksheet 3.A3.B “Initial Amplification...” record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples to be processed and the amount of MM needed per reaction (40 µl). Add 4 to the number of samples so that there is enough to run controls.
  3. Under UV Cabinet, add PCR reagents **except for sample DNA** to the MM tube in the order listed on Worksheet 3.A3.B, adding water first and Taq last. Keep all reagents cold during mixing, and return them to freezer immediately after use. Do not expose enzymes, Primers, or dNTP’s to UV light.
    - a. Water to make a 40 µl total volume per reaction
    - b. PCR Buffer mix (1X)
    - c. MgCl<sub>2</sub> (1.5 mM per reaction)
    - d. dNTP mix (0.2 mM per reaction)
    - e. Primers (20 pmole each per reaction)
      - i. Forward 5’ - A GCT TCG CAA GGT GAA GGG – 3’
      - ii. Reverse 5’ – GC AAC AGG TTT ATT TGC CGG G – 3’
    - f. TAQ polymerase (2 units per reaction)
  4. Place 40 µl of MM into each 0.5ml PCR tube and close caps tightly. Move PCR tubes to sample loading area.
  5. In sample loading area, load 10µl of each sample DNA to the appropriately labeled PCR tubes. Close caps tightly. Remove sample tubes from UV cabinet to thermocycler.
  6. Load the sample tubes into the thermocycler wells.

7. Thermocycler should be programmed for 30-40 cycles of the following temperature regime, and recorded on Worksheet 3.A3.B:

Preheat sample to 94°C for two minutes.  
Denaturing at 93°C for 30 seconds.  
Annealing at 60°C for 30 seconds.  
Extending at 72°C for 1 minute.  
Post dwell at 4-16°C for holding  
samples after cycling is complete.

- c. “Nested” PCR-secondary amplification of *R. salmoninarum* DNA - Materials, methods and general QA/QC considerations of this section and Chapter 6 also apply to the nested PCR process:
  - i. Using Worksheet 3.A3.C “Nested PCR” record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples and controls to be processed and the amount of MM needed for each reaction (49 µl).
  - ii. Add PCR reagents except the first round amplified DNA into the Master Mix (MM) tube. Return reagents to freezer
    - I. Primers:
      - a. Forward 5’ – AT TCT TCC ACT TCA ACA GTA CAA GG – 3’
      - b. Reverse 5’ – C ATT ATC GTT ACA CCC GAA ACC – 3’
  - iii. In PCR tubes (0.5 ml), pipette 49µl of MM. Close caps tightly. Remove tubes from UV cabinet to amplified DNA area
  - iv. Load 1µl of amplified sample DNA into the appropriate PCR tubes
  - v. Load PCR tubes into thermocycler wells
  - vi. Program thermocycler for 10-20 cycles of the following regime:

Preheat sample to 94°C for two minutes.  
Denaturing at 93°C for 30 seconds.  
Annealing at 60°C for 30 seconds.  
Extending at 72°C for 1 minute.  
Post dwell at 4-16°C for holding  
samples after cycling is complete.

**Note:** PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

- d. Visualization of PCR Product by Electrophoresis – See Chapter 6 for general procedures.
  - i. Visualization of amplified products resulting from PCR for detection of *R. salmoninarum* DNA is best accomplished after electrophoresis on a 1.5 or 2% agarose gel (6.3.C)
  - ii. Using Worksheet 3.A3.C “Gel Preparation and Lane Designation”, record location of each sample on the agarose gel at the time samples are loaded.
  - iii. After electrophoresis, stain gel with ethidium bromide, and visualize on an UV transilluminator.
  - iv. Carefully record locations of bands on positive control samples in relation to DNA ladder bands.
    - 1. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays (first round primer M21=383bp; nested primer M38=320 bp). **Bands occurring at these locations are confirmatory for *R. salmoninarum* and are reported as POSITIVE.**
    - 2. Note any unusual band occurrences. Negative controls should not have any bands. Suspicion of contamination indicates PCR should be re-run on samples from template DNA tube.
- e. Document the electrophoresis results (6.3.G) - photograph all gels and attach the photo to Worksheet 3.A3.D. Attach to case history information.

### **3.6 Reagents, Media and Media Preparation**

**A. Growth Media** – Most bacteriological culture medias are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Recipes for those media which are not available in a commercial preparation are included below:

1. **Brain Heart Infusion Agar (BHIA)** (Difco 1998) - A basic agar for most bacterial cultures.
2. **Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB)** (Difco 1998) – A basic media for most bacterial cultures.
3. **Selective Kidney Disease Medium-2 (SKDM-2)** (Austin, et. al. 1983) - for selective isolation of *Renibacterium salmoninarum*

Peptone	10	gm
Yeast Extract	0.5	gm
L-Cysteine HCL	1	gm
Agar	15	gm
Distilled Water	874	ml

Adjust pH to 6.5 before adding agar. Autoclave for 15 minutes at 121°C. Cool to ~ 50°C and add:

Fetal Bovine Serum	100.0 mls
--------------------	-----------

The following antibiotics are added to reduce overgrowth from other bacterial organisms):

4.0 mls Cyclohexamide (1.2 gms Cyclohexamide in 96 mls dH<sub>2</sub>O)  
1.0 ml D-Cycloserine (0.3 gms D-Cycloserine in 24 mls of dH<sub>2</sub>O)  
2.0 mls Polymyxin B-sulfate (0.3 gms Polymyxin B-sulfate in 24 mls of dH<sub>2</sub>O)  
1.0 mls Oxolinic Acid (0.06 gms Oxolinic Acid in 24 mls of 5% NaOH)

**B Media to Identify Growth and Biochemical Characteristics** - Most of these media are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Unless otherwise specified, these media can also be prepared from basic laboratory ingredients according to recipes found in the following references: MacFaddin 1980, MacFaddin 2000, and Difco 1998.

1. **Motility Test Medium** - A semi-solid media used as a tube test to detect the ability of a microorganism to exhibit motility. Several types are commercially available, including MIO (motility, indole, ornithine) which allows for the detection of motility, and the reaction of two biochemical tests in the same tube.

2. **Tryptic soy broth( TSB)** – a nutrient broth media used to determine motility of a microorganism with the hanging drop method.
3. **Triple sugar iron agar (TSI)** – A commercially prepared dehydrated media used to evaluate the utilization of glucose and two additional carbohydrates, as well as the production of hydrogen sulfide.
4. **Oxidation/Fermentation (OF) medium** – A basal media for Carbohydrate Utilization Tests, available in a commercially prepared dehydrated powder. The OF basal is prepared according to manufacturer's recommendations prior to the addition of individual carbohydrates as described below:

To prepare final medium aseptically add 10 ml of a filter-sterilized (0.45 µm) 10% carbohydrate solution to autoclaved and cooled (50°C) media resulting in a 1% final concentration, with the exception of salicin, which should be made as a 5% solution resulting in a 0.5% final concentration(see below). Only one carbohydrate is added to the basal medium for each test to be run.

10% Arabinose (1 gm Arabinose to 10 ml in dH<sub>2</sub>O)  
 10% Rhamnose (1 gm Rhamnose to 10 ml in dH<sub>2</sub>O)  
 10% Sucrose (1 gm Sucrose to 10 ml in dH<sub>2</sub>O)  
 10% Sorbitol (1 gm Sorbitol to 10 ml in dH<sub>2</sub>O)\*  
 5% Salicin (0.5 gm Salicin to 10 ml in dH<sub>2</sub>O)

Mix flask thoroughly and aseptically dispense into sterile tubes. Store at 2-8°C. Final pH = 6.8 ± 0.2 at 25°C.

\*A sorbitol utilization slant media can also be prepared and utilized as described in Cipriano and Pyle, 1985.

5. **Nutrient Gelatin** – A dehydrated medium for determining the presence of Gelatinase.
6. **Tryptone Broth** – for use with the Indole test.

Tryptone	10 gm
Distilled Water	1000 ml

Heat gently to dissolve. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Store at 2-8°C.

7. **Decarboxylase Medium Base** – A basal media for use in Lysine test.

The basal media, without addition of lysine, serves as the control.

To make L-Lysine media add 5 gm L-Lysine to 1 liter of prepare basal

decarboxylase media. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Final pH =  $6.8 \pm 0.2$  at 25°C. Store at 2-8°C.

8. **Malonate Broth** – A media used for the malonate test.
9. **Bile Esculin Agar** – A media used for the esculin Test

## C Media Preparation

### 1. **Plate Media**

- a. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely suspend agar. Use of a stir bar will facilitate mixing of agar.
- b. Cover beaker with foil, or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121°C for 15 minutes at 15 pounds pressure (consult with the manual provided by the autoclave manufacturer for adjustment of time when large volumes of media are being sterilized).
- c. Cool media to 50°C.
- d. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date and initials. When media is needed, boil, microwave or use a water bath to completely melt the agar. Cool to 50°C.
- e. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface. Mix any added ingredients into the media at this temperature.
- f. Label the bottom of each plate with medium type and date prepared.
- g. Remove bottle cap and pour plates or dispense with a sterile Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 ml per 100 X 15mm petri dish. Replace lids as soon as the plate is poured.
- h. Invert plates when the media has cooled completely (~ 30-60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.
- i. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.
- j. Follow manufacturer's recommendation for storage period of prepared media. Each batch should be labeled with date of preparation.

## 2. Tube Media

- a. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely suspend the agar. Use of a stir bar will facilitate mixing of agar.
- b. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise compensation for temperature needs to be made.
- c. Arrange test tubes in racks. Disposable, autoclavable screw cap tubes can be used for all tube media.
- d. Use an automatic pipetter or Pipette-aid™ to dispense the medium. If using the Brewer or Cornwall pipette prime with deionized water, then pump the water out of the syringe prior to pipetting and discard the first few tubes of media that are dispensed. Dispense approximately 5 to 10 ml media in 16 X 125mm or 20 X 125mm tubes. Close caps loosely.
- e. Immediately after use, rinse the automatic pipetter in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.
- f. Loosely place screw caps on tubes. Do not tighten caps. It is necessary to allow pressure to release from tubes while heating in the autoclave.
- g. Follow manufacturer's recommendation for autoclave time and temperature.
- h. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. long butt and short slant for TSI or a standard slant over  $\frac{3}{4}$  of the tube length for TSA or BHIA). Then tighten caps.
- i. Cool completely to room temperature in the slanted position.
- j. Label the tubes or the tube rack with type of medium and date made.
- k. Store at 2-8°C, following manufacturer's recommendation for period of long-term storage.

**D Reagents**-Most of these reagents are commercially available pre-made. These commercial products are entirely acceptable and should be stored according to the manufacturer's recommendations. The formulations provided below were obtained from the references cited.

1. **Gram Stain Reagents** - These stains can be ordered as a complete kit or can be prepared as follows:

a. Crystal violet

crystal violet (90% dye content)	20.0 g
ethanol (95%)	200 ml
ammonium oxalate	8.0 g
dH <sub>2</sub> O	800 ml

Mix first two ingredients and let sit overnight or until dye goes into solution. Add remaining ingredients and filter before use.

b. Gram's iodine

iodine crystals	1.0 g
potassium iodide	2.0 g
dH <sub>2</sub> O	300 ml

c. De-colorizer

acetone	40 ml
ethanol (95%)	60 ml

d. Safranin

safranin O	2.5 g
95% ethanol	100 ml
dH <sub>2</sub> O	900 ml

Filter safranin solution before use.

2. **FAT Mounting Fluid (pH 9.0):**

Glycerol	90.0 ml
DABCO*	2.5 g
PBS	10.0 ml

Suspend the DABCO in glycerol over low heat. Then add 1X PBS (see below). Adjust pH to 8.6 - 9.0 with 1N Hydrochloric Acid or 0.1N Sodium Hydroxide. The pH of the mounting media is important, as an acid pH will quench fluorescence. Check the pH frequently. Store at room temperature.

\* Optional ingredient - DABCO is 1,4-diazabicyclo-(2,2,2)-octane. Its addition to mounting fluid can reduce quenching of fluorescence.

3. **Phosphate-buffered saline for FAT (PBS), pH 7.1**

- a. 1× concentration (0.15 M NaCl, 0.01 M phosphate; makes 1 L)
- |   |        |
|---|--------|
| NaCl .....  | 8.50 g |
| Na <sub>2</sub> HPO <sub>4</sub> (anhydrous)..... | 1.07 g |



NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O (monohydrate)	0.34 g
DH <sub>2</sub> O .....	to 1 L

Adjust pH to 7.1 with 1N Hydrochloric Acid or 0.1N Sodium Hydroxide.

- b. 5× concentration (makes 10 L of 1× PBS )
- |  |         |
|--|---------|
| NaCl .....   | 85.00 g |
| Na <sub>2</sub> HPO <sub>4</sub> (anhydrous).....                | 10.70 g |
| NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O (monohydrate) | 3.45 g  |
| DH <sub>2</sub> O .....  | to 2 L  |

Adjust pH to 7.1 with 1N Hydrochloric Acid or 0.1N Sodium Hydroxide.

4. **Kovac's Indole reagent**

Isoamyl alcohol	30 ml
p-Dimethyl aminobenzaldehyde	2 g
Hydrochloric Acid (HCl)	10ml

Dissolve the aldehyde in the alcohol. Slowly add the acid to the mixture. Store solution at 2-8°C in amber dropper bottle.

5. **Counter Stains**

- Rhodamine - Rehydrate the rhodamine to 1 mg/ml in distilled water. If the rhodamine does not completely dissolve, add a small drop of 0.1 M Sodium Hydroxide. Store at 2-8°C
- Evans Blue – Prepare 0.1% stock solution with distilled water and decontaminate with 0.45 µm membrane filtration. Store at room temperature. Prepare a 0.01% working dilution with sterile PBS (3.6.D.3). (Cvitanich, 1994)
- Eriochrome black T – prepare a solution at 1:60 (w/v) in PBS (3.6.D.3), and filter through Whatman #1 and Whatman # 42 filter papers before initial use (Elliott & McKibben, 1997)

6. **FITC Conjugated Rabbit Anti-X /Rhodamine Counter Stain**

Rhodamine stock	10.0 µl
FITC conjugated antibodies	10.0 µl
Phosphate buffered saline	480.0 µl

Store at 4°C.

**Note:** Evans Blue or Eriochrome black T can also be used as counterstain in FAT in a separate step during the staining process, but neither can be added directly to the conjugate solution.

**E. Cytochrome Oxidase Spot Test** - Individual test strips can be purchased from several suppliers (catalogue # 38-191, Remel, Tel. 800-255-6730).

**F. Determination of Antiserum and Conjugate Working Dilutions for FAT**

Commercially prepared anti-sera and conjugates are should be reconstituted according to the manufacturer's instructions. Aliquots of 0.5 ml can be frozen for later dilution into a working solution of the reagent. Reagents are more stable if frozen as a stock solution. The proper working dilution is the highest dilution that still maintains maximum brightness of the fluorochrome on positive control FAT preparations. Generally the manufacturer will recommend between 1:20 to 1:50 using a suitable buffer (PBS) as the diluent. However, in all cases each laboratory must establish the proper working dilution by starting with the manufacturer's recommendation and bracketing, or testing dilutions on either side of the recommended concentration. The following example shows how to determine the correct working dilution of FAT conjugate where the manufacturer recommends a working dilution of 1:40.

1. Using the stock solution, dilute the antiserum at 1:20, 1:30, 1:40, 1:50, 1:60 using PBS or another buffer as recommended by antibody manufacturer.
2. Rhodamine counter stain is added directly to the optimum antiserum working dilution at a 1:50 (alternatively, Evan's Blue may be used to counterstain FAT stained slides – do not add Evan's blue to the conjugate directly).
3. The FAT is performed on replicates of a known positive control, each replicate using a different dilution of the conjugated antiserum. In this way the working dilution can be determined as the endpoint of optimum fluorescence (the highest dilution that still provides a bright specific fluorescence with little or no background staining).
4. Prepared conjugate solutions should be filtered through 0.2 to 0.4 micron filter prior to use and storage. Store frozen in small aliquots. Do not thaw and re-freeze antibodies repeatedly.

**G. PCR Reagents** – the following are formulations for extraction reagents used specifically in the protocols described in 3.5.E.2.

1. **Lysozyme lysis buffer** – 100 mLs (this formulation is used for lysis of gram positive bacteria with Qiagen extraction kits)

Lysozyme	2 g
Tris HCl Stock	2 mLs
EDTA stock	2 mLs

Triton	1.2 mL
--------	--------

Bring these components to 100 mL with sterile distilled water (molecular grade)

2. **Tris HCl Stock solutions** – 100 mLs at 1M pH 8.0 (for use in lysozyme buffer)

Trizma base	5.7 g
Tris HCl	8.9 g
dH <sub>2</sub> O	85.4 mL

3. **EDTA Stock solution** – 100 mLs at 0.1 M (for use in lysozyme buffer)

EDTA	3.72 g
------	--------

Bring to 100 mL with sterile distilled water (molecular grade).

### **3.7 Bacterial Identification Techniques**

Unless otherwise specified, all of the materials and techniques described in Section 3.7 are described in detail in MacFaddin's (2000) and or the 11<sup>th</sup> Edition Difco Manual (1998). Each of the tests listed are provided with a set of control bacterial species available from ATCC, which will provide quality control for each biochemical test. It is not necessary, however, to set up control isolates for every test run in these protocols. It is strongly suggested that newly prepared batches of media and reagents be tested using the control bacterial isolates listed for each.

**A. Gram Reaction** - Gram staining detects a fundamental difference in the cell wall composition of bacteria.

1. **Gram Stain:** (Kits are available commercially, or formulas for reagents are listed in 3.6.A.)
  - a. Prepare a bacterial smear from a pure culture
    - i. Put a drop of saline, distilled water, PBS (3.6.D.3), or formalin saline (0.4% formalin, 0.85% NaCl) on a clean glass slide
    - ii. Using a sterile loop or needle touch an isolated colony and mix in the water drop.
    - iii. Mix until just slightly turbid (light inoculum is best, excess bacteria will not stain properly).
    - iv. Let air dry and heat fix. Do not overheat; slide should not be too hot to touch.
    - v. Allow to cool.
  - b. Flood the slide with crystal violet (3.6.D.1.a), and allow to remain on the slide for 60 seconds
  - c. Wash off the crystal violet with running tap water.
  - d. Flood the slide with Gram's iodine (3.6.D.1.b), and allow to remain on the slide for 60 seconds.
  - e. Wash off with running tap water.
  - f. Decolorize with decolorizer solution (3.6.D.1.c) until the solvent flows colorless from the slide (approximate 5-10 seconds). Excessive decolorization should be avoided since it may result in a false gram-negative reading. Too little decolorization can result in a false positive result.
  - g. Rinse immediately with running tap water.

- h. Counter stain with Safranin (3.6.D.1.d) for 60 seconds.
- i. Rinse with tap water and allow to air dry.
- j. RESULTS:
  - i. Gram negative: cells are decolorized by the alcohol-acetone solution and take on a pink to red color when counter stained with safranin.
  - ii. Gram positive: cells retain the crystal violet and remain purple to dark blue.
- k. Quality Control: Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2-8°C for long term use)
  - i. Positive: *Staphylococcus sp.* (ATCC – any isolate)
  - ii. Negative: *Yersinia ruckeri*
  - iii. Commercially prepared Gram stain control slides are available (Fisher Scientific, #08-801)

2. **3% Potassium Hydroxide** - Alternative test for Gram reaction

- a. Add a heavy inoculum of a pure culture of bacteria grown on a solid medium to a drop of 3% potassium hydroxide (KOH) solution (3 grams KOH per 100 mls distilled water) on a clean glass slide.
- b. Stir for about one minute, occasionally lifting the loop to look for thickening and “stringing” of the slurry.
- c. RESULTS:
  - i. Gram-positive bacteria will not appear to change the viscosity of the KOH solution.
  - ii. Gram negative bacteria will cause the KOH solution to become stringy or mucoid in appearance and consistency
- d. QUALITY CONTROL: Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2-8°C for long term use)
  - i. Gram Positive: *Staphylococcus sp.*
  - ii. Gram Negative: *Yersinia ruckeri*

**B. Cytochrome oxidase (3.6.E)** – this test determines the presence of cytochrome oxidase enzymes. The use of an iron-containing metal inoculation loop can lead to a false-positive reaction. Use only plastic or platinum loops for this test.

1. Add an inoculum of a pure 18-24 hour old bacterial culture to the surface of the test strip impregnated with reagent.
2. Results:
  - a. Positive: purple color within 5-10 seconds (reactions that occur after 10 seconds are negative).
  - b. Negative: no purple color.
3. Quality Control:
  - a. Positive: *Pseudomonas aeruginosa* (ATCC 10145)
  - b. Negative: *Yersinia ruckeri*
  - c. Observe expiration dates of reagent strips.

**C. Motility** – this test determines if a bacterial isolate is motile by means of flagella.

1. Hanging Drop Method
  - a. Inoculate a tryptic soy broth (TSB (3.6.A.2)) with the isolate.
  - b. Incubate at room temperature until growth is obtained, usually 24 hours.
  - c. After incubation use a sterile loop or sterile dropper and place a drop on a clean cover slip.
  - d. Place a tiny drop of distilled water in one corner of the same cover slip.
  - e. Carefully invert the cover slip and place over the concave portion of a hanging drop slide.
  - f. Observe for motility at 400X magnification on a compound microscope. Care should be taken to not interpret “drift” or “Brownian motion” as motility.
  - g. Record results as motile or non-motile.
2. Semi-Solid Medium Method (3.6.B.1)
  - a. Stab the semi-solid medium with a small amount of inoculum.
  - b. Incubate overnight at room temperature.
  - c. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

- d. Confirmation of results using the hanging drop method is recommended.
3. Quality Control:
    - a. Positive: *Escherichia coli* (ATCC 25922)
    - b. Negative: *Aeromonas salmonicida*

## D. Biochemical Testing

### 1. Tube Method

- a. **Glucose Fermentation** - An OF basal medium (3.6.B.4) is used to test the fermentation of glucose by bacterial isolates.
  - i. With a sterile needle inoculate two tubes of OF-glucose by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.
    1. Fermentation Test - One tube is over-layed with sterile mineral oil or paraffin. Sterile petroleum jelly (heated to melting) should be used for more accurate observation of gas production.
  2. Oxidation Test – The second tube is not overlaid.
- ii. Incubate at 20-24°C and read after 18-24 hours.
- iii. Results:
  - A = Acid (yellow)
  - AG = Acid + Gas
  - N = No change or Alkaline (green or blue-green)

	<u>Fermentation Test Tube</u>	<u>Oxidation Test Tube</u>
Fermentative	A or AG	A or AG
Oxidative	N	A or AG
Non-reactive	N	N

- iv. Quality Control:
  1. Fermentative: *Aeromonas* species
  2. Oxidative: *Pseudomonas fluorescens*
- b. **Triple Sugar Iron (TSI)** - TSI agar (3.6.B.3) contains the three sugars in varying concentrations: glucose (1X), which is a simple monosaccharide, and lactose and sucrose (10X each), both of which are disaccharides. It also contains the pH indicator phenol red. All organisms that utilize glucose will yield an initial acidic reaction throughout the tube (yellow - see below) regardless of whether they utilize sucrose or lactose. Reversion will not occur until all the glucose in the slant portion is completely utilized. At this point, the bacterium will utilize either one or both of the disaccharides, continuing the

production of acids in the media (the slant remains yellow). If, after all the glucose in the slant is used and the bacterium cannot utilize either lactose or sucrose, the bacterium is forced to revert to protein (peptone) present in the agar. In this case, nitrogenous bi-products are produced and the pH in the media rises until the pH indicator shows a reversion from yellow (acid) to red (alkaline). Blackened medium is caused by hydrogen sulfide production, which changes ferrous sulfate to ferrous sulfide. In addition, splitting of the medium or presence of bubbles in the butt of the tube can determine gas production.

- i. With a sterile needle inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.
- ii. Incubate at 20-24°C. Read after 18-24 hours.
- iii. Results:
  - A = Acid
  - K = Alkaline
  - H<sub>2</sub>S = Hydrogen sulfide produced
  - N = No change

Slant / Butt	Color	Interpretation
K / N or K / A	Red / Orange	Only Peptone Utilized
	Red / Yellow	Only Glucose Fermented
A / A	Yellow / Yellow	Glucose plus Lactose and/or Sucrose Fermented
Gas	Splitting or Bubbles	Gas Production
H <sub>2</sub> S	Black Butt	Hydrogen Sulfide Produced

- iv. Quality Control:
  1. K/A: *Shigella flexneri* (ATCC 12022)
  2. A/AG: *Escherichia coli* (ATCC 25922)

c. **Gelatinase**– A test to determine bacterial production of gelatinases, enzymes that liquefy gelatin.

- i. Inoculate by stabbing ½ to 1 inch deep into the Nutrient Gelatin media (3.6.B.5) with a heavy inoculum from an 18-24 hour pure culture.
- ii. Incubate 18-24 hours at 20°C.
- iii. Results:
  1. Positive (+) - Media is liquefied. Weak results can be visualized by rapping the tube against the palm of the hand to dislodge droplets of liquid from the media. Any drops seen are considered positive.
  2. Negative (-) - No liquefaction occurs in media.



iv. Quality Control:

1. Positive: *Proteus vulgaris* (ATCC 8427)
2. Negative: *Escherichia coli* (ATCC 25922)

v. Precautions:

1. The liquid will generally appear turbid due to bacterial growth.
2. Nutrient Gelatin softens at temperatures above 20°C. Keep refrigerated until ready to inoculate, and do not let tubes reach room temperature or warmer. This will make interpretation of results difficult. Tests, which are incubated at 35°C, should be refrigerated prior to recording results.

d. **Indole Test** - A test to determine bacterial ability to split indole from the tryptophan molecule. Certain bacteria are able to oxidize the amino acid, tryptophan, with tryptophanase enzymes to form three indolic metabolites - indole, skatole (methyl indole) and indoleacetate. Indole, pyruvic acid, ammonia, and energy are principle degradation products of tryptophan. Indole, when split from the tryptophan molecule, can be detected with the addition of Kovac's reagent. The reagent is not a dye or stain, but reacts with indole to produce an AZO dye.

- i. Inoculate tryptone broth (3.6.B.6) with a light inoculum from an 18-24 hour pure culture.
- ii. Incubate 24 to 48 hours at 20°C
- iii. At the end of 24 hours incubation –
  1. Aseptically remove 2 ml of media and place in an empty sterile test tube. Save extra tube for 48-hour incubation, if necessary.
  2. Add about 5 drops of Kovac's reagent (3.6.D.4) to one of the tubes and agitate tube.
  3. If a positive reaction is observed, the test is complete.
  4. If the 24 hour incubated sample is negative, incubate the remaining tube for an additional 24 hours, and test again for the presence of indole with Kovac's reagent.

iv. Results:

1. Positive (+) - within 1 to 2 minutes a cherry red ring will form at the surface of the media.
2. Negative (-) - No color formation is observed at the surface, the

color remains that of the reagent – yellow.

3. Variable (V) - An orange color may develop. This indicates the presence of skatole, which may be a precursor of indole formation.

v. Quality Control:

1. Positive: *Escherichia coli* (ATCC 25922)
2. Negative: *Pseudomonas aeruginosa* (ATCC 27853)

vi. Precautions:

1. Avoid inhaling fumes of Kovac's. Wear gloves to avoid skin contact.
2. Tests for indole should be conducted after both 24 and 48 hours of incubation before a test can be declared negative. Split the broth culture prior to performing the 24-hour test. If negative, incubate the untested tube (without Kovac's) for another day and try again.
3. Do not eliminate the 24-hour test, because some organisms may have produced indole by 24 hours, but have broken it down by 48 hours. DO BOTH!
4. Kovac's reagent should be fresh. A color change from yellow to brown indicates aging and results in reduced sensitivity of the test.
5. The procedure described here produces more reliable results than those obtained from MIO (motility-indole-ornithine) medium.

- e. **Carbohydrate Utilization** (MacFaddin 1980)- the following carbohydrates are utilized to aid in bacterial species identification: Arabinose, Rhamnose, Mannitol, Salicin, Sorbitol, and Sucrose (saccharose). The procedures to be followed for each of these media are identical.

- i. Inoculate carbohydrate tube (3.6.B.4) with growth from an 18-24 hour pure culture.
- ii. Incubate with loosened cap 18-24 hours at 20°C. A prolonged incubation of up to 4 days may be necessary for some negative results.
- iii. Results:

1. Positive (+) - Acid is produced from fermentation, which turns media yellow.
2. Negative (-) - No fermentation of carbohydrate, media remains green.
3. Aerogenic (G) - Gas bubbles are present within the media.

iv. Quality Control:

<u>Carbohydrate</u>	<u>Positive Control Isolate</u>	<u>Negative Control Isolate</u>
Arabinose	<i>Escherichia coli</i> (ATCC 25922)	<i>Yersinia ruckeri</i>
Sorbitol:	<i>Escherichia coli</i> (ATCC 25922)	<i>Y. ruckeri</i> Type I
Rhamnose	<i>Enterobacter aerogenes</i> (ATCC )	<i>Yersinia ruckeri</i>
Salicin	<i>Enterobacter aerogenes</i> (ATCC )	<i>Yersinia ruckeri</i>

v. Precautions:

1. Difficulty in interpreting test results may occur with slow growing bacteria. Prolonged incubation may be required.
2. Heavy bacterial growth throughout the media can offset the color of a negative (green) reaction, giving the appearance of a weakly positive (yellow) reaction. This is especially true with yellow-pigmented bacteria. These tubes should be retested if a true yellow color is not noted within several days.

f. **Decarboxylase Test (Lysine)** - A determination of bacterial enzymatic capability to decarboxylate an amino acid to form an amine with resultant alkalinity.

- i. For each isolate to be tested, it is necessary to inoculate a Decarboxylase control tube and Lysine test tube (3.6.B.7). Use light inoculum from 18-24 hour pure culture.
- ii. Add 1-2 ml oil overlay to each tube.
- iii. Incubate 24 hours at 20°C. A prolonged incubation of up to 4 days may be necessary.

iv. Results:

<u>Test Result</u>	<u>Lysine Tube</u>	<u>Control Tube</u>
Positive	Turbid to faded purple (glucose fermented, decarboxylase produced)	Yellow (glucose fermented)
Negative	Yellow (glucose fermented)	Yellow
Negative	Purple (glucose not fermented, decarboxylase not produced)	Purple (glucose not fermented)

v. Quality Control:

1. Positive: *Yersinia ruckeri*
2. Negative: *Enterobacter cloacae* (ATCC 13047)

vi. Precautions:

1. At the end of incubation, the Lysine tube might show a layer of purple over yellow. Gently shake the tube before interpreting the result.
2. An indistinct yellow-purple color may be difficult to interpret. Use the control tube for comparison. Any trace of purple color after a 24-hour incubation in the amino acid tube denotes a positive result.
3. Do not interpret tests prior to 18-24 hours. During the first 12 hours, only glucose is fermented which produces a yellow color. Decarboxylase enzymes do not form until the acidic environment is established by the fermentation of glucose.

g. **Malonate Test** - A method to establish if a bacterial isolate is able to utilize sodium malonate as its only source of carbon.

- i. Inoculate malonate media (3.6.B.8) with a light inoculum from an 18 - 24 hour pure culture.
- ii. Incubate 24 to 48 hours at 20°C.

iii. Results:

1. Positive (+) - light blue to deep blue color throughout the media.
2. Negative (-) - color remains the same as un-inoculated tube - green.

- iv. Quality Control:
  - 1. Positive: *Enterobacter aerogenes* (ATCC 13048)
  - 2. Negative: *Yersinia ruckeri*
- v. Precautions: The test tube must be incubated for at least 48 hours before it may be called negative. Since some bacteria produce only slight alkalinity, it is useful to compare the test to an un-inoculated tube. Any trace of blue color denotes a positive reaction.

h. **Esculin Test** - To determine the ability of an organism to hydrolyze the glycoside esculin (aesculin) to esculetin (aesculetin) and glucose in the presence of bile (10 to 40%).

- i. Inoculate the surface of the bile esculin slant (3.6.B.9) with inoculum from an 18 to 24 hr. old pure culture
- ii. Incubate 20° C for 24 to 48 hrs.
- iii. Results:
  - 1. Positive (+): presence of a black to dark brown color on the slant.
  - 2. Negative (-): No blackening of the medium.
- iv. Quality Control:
  - 1. Positive: *Enterobacter aerogenes* (ATCC 13048)
  - 2. Negative: *Yersinia ruckeri*
- v. Precautions: False positives may occur with hydrogen sulfide producing organisms, such as *Shewanella putrefaciens*. Neither of the target organisms for these protocols will, however, produce hydrogen sulfide.

2. **Commercial Identification Systems** – Several commercial test strips or kits are available for biochemical testing of bacteria. Bear in mind that these kits are designed for human and/or animal testing and the manufacturer's recommended incubation temperature is 37°C. The decreased incubation temperature (22°C- room temperature) required for most fish pathogens results in slightly different reactions and longer incubation periods. Therefore, test results may not follow the manufacturer's identification profiles exactly. Therefore, it may be necessary to refer to the Charts in 3.A2 for identification when API strips are employed.

- a. **API 20E™** – The API 20E™ system is a standardized, miniaturized version of conventional procedures for the identification of *Enterobacteriaceae* and other Gram-negative bacteria. It is a microtube system designed for the performance

of 23 standard biochemical tests from isolated colonies on plating medium. Refer to the instructions enclosed with each kit for more detailed information. The API system™ is available from bioMérieux (1-800-638-4835, catalog #20-109/20-179). Reference charts of API profiles for *A. salmonicida* and *Y. ruckeri* are listed in 3.A2.

- b. **Biolog** – MicroLog™ is a microbial identification system able to identify and characterize a wide variety of organisms based on carbon source utilization. The system has identification databases that contain over 1400 different species/genera of aerobic and anaerobic bacteria and yeasts. The identification databases include a wide variety of organism including animal, plant, and water pathogens. The system also allows the user the capability to build customized organism databases. Products are available directly from Biolog, 3938 Trust Way, Hayward, CA. 94545 (1-510-785-2564 or website [www.biolog.com](http://www.biolog.com)).

**E. Fluorescent Antibody Test (FAT)** - The Fluorescent Antibody Test (FAT) is one serological method for corroboration testing of bacterial isolates. FAT can be performed either with a direct antibody staining (DFAT) or indirect (IFAT) technique, depending upon the availability of pathogen-specific FITC-conjugated or unconjugated antibody preparations from the manufacturers listed below (3.7.E.6). There are three basic steps for FAT: preparing and fixing bacterial samples; staining the slides with antibody reagents; reading and interpreting the slides.

1. Preparing the slides

- a. Pure Bacterial Cultures (confirmatory testing of pure isolates of *A. salmonicida*, *Y. ruckeri*, or *E. ictaluri*).
  - i. Pure isolates of bacteria are diluted in sterile PBS (3.6.D.3) and applied to 2 replicate wells of an FAT slide.
  - ii. Allow air-drying completely, or heat fix.
- b. Kidney (for presumptive detection of *R. salmoninarum*) – prepare kidney smear on a non-coated or acetone-cleaned glass slide
  - i. Homogenize a piece of posterior kidney tissue and create a thin smear on the surface of a slide.
  - ii. Allow to air dry completely, or heat fix.
- c. Ovarian Fluid pellet smear (DFAT-for presumptive detection of *R. salmoninarum*) – ovarian fluid is collected (2.2.E.2.f).
  - i. After pooled ovarian fluid samples are processed and appropriate amount of supernatant removed for virology assays (4.4.C), the pellet is re-suspended in the remaining supernatant by thorough vortexing or repeat pipetting.

ii. Transfer two 1.5 mL aliquots from each pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL micro-centrifuge tubes (see **Note**). Freeze the remainder of the sample at -20°C for PCR confirmation.

iii. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes.

**Note:** Elliot and McKibben (1997) document the importance of using a minimum of 10,000 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e. 2.5 mLs from a 5-fish-pool). Most 15 mL centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal force (rcf) as high as 10,000 X g. It is, therefore, necessary to aliquot the appropriate amount of fluid into microcentrifuge tubes suitable for the required rcf.

iv. The pellet is carefully and a thin smear prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

v. Allow to air dry completely, or heat fix.

d. After the tissue is completely air dried or heat fixed, slides are fixed in acetone for 5 minutes. Other fixing solutions are suitable as long as a component of the solution contains a lipid dissolving reagent such as acetone or xylene, which helps remove lipids and improve the overall fluorescence quality and intensity.

2. **Direct FAT (DFAT) Staining** (Thoesen, 1994)- Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or tissue).

a. Positive and Negative Controls: Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for FAT staining. Positive controls are always used in confirmation testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

b. Place slides in dark, humidified chamber, and place one drop of specific FITC conjugate (3.6.D.6) on each sample slide and control slides.

c. Incubate for 30-60 minutes at room temperature, according to manufacturer's recommendation.

- d. Using a squirt bottle or transfer pipette, GENTLY rinse the slides with PBS (3.6.D.3).
  - e. If Rhodamine counterstain has not been incorporated in the FAT stain, apply the counterstain of choice (3.6.D.5) at this point for 1-2 minutes.
  - f. Final rinse/soak in PBS for 5-10 minutes. Air-dry completely.
  - g. Add a small drop of FA Mounting Fluid, pH 9 (3.6.D.2) to each slide, being careful not to touch the dropper to the slide to prevent the possibility of cross contamination.
  - h. Place a 24 x 50 mm cover slip over the slide using care not to trap air bubbles.
  - i. Spread the mounting fluid by gently pressing the cover slip with the blunt end of a pen or lab marker.
  - j. Add one drop of immersion oil to the cover slip and examine at 1000X using the epifluorescent filter.
3. **Indirect FAT (IFAT) Staining** (Thoesen, 1994) – IFAT is a double layered antibody technique, where the first layer consists of unconjugated purified, immunoglobulin (IgG) or antibody prepared in one animal species (e.g. rabbit) against the target antigen. The second antibody applied is a FITC-conjugated antibody prepared in a second animal species (e.g. goat), and specific for IgG of the first animal species (e.g. goat anti-rabbit IgG). Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or tissue).
- a. Positive and Negative Controls: Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for FAT staining. Positive controls are always used in corroboration testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.
  - b. Place slides in dark, humidified chamber, and place one drop of unconjugated, pathogen specific antibody on each sample slide and control slides.
  - c. Incubate at room temperature for 30-60 minutes, according to manufacturer's recommendations.
  - d. Gently rinse slides with PBS (3.6.D.3).



- e. Place a drop of FITC-conjugated second antibody on slides and incubate at room temperature in dark chamber according to manufacturer's recommendation.
- f. Rinse briefly with PBS. If rhodamine counterstain as not been incorporated into the FAT stain, apply the counterstain of choice (3.6.D.5) at this point for 1-2 minutes.
- g. Rinse and wash in PBS for 5-10 minutes.
- h. Air dry completely and apply a small drop of FA mounting fluid, pH 9 (3.6.D.2). Apply a cover slip to mounting fluid.
- i. Add one drop of immersion oil to the coverslip and examine at 1000X using the epifluorescent filter.

#### 4. Reading Results:

- a. Slides are read at 1000x on a compound fluorescence microscope (refer to the microscope manufacturer for correct wavelength and filters required for FITC epifluorescence microscopy). The positive and negative control slides are read first. This has two purposes: (1) quality assurance for the staining process (positive control should have myriad numbers of fluorescing bacteria, the negative control should have no fluorescence); and (2) to familiarize the reader with the correct bacterial size, shape, and magnitude of the fluorescent halo of bacteria in the positive control. The reader can refer back to the positive control as a reference, if needed, to confirm suspect bacteria in the sample wells. Positive fluorescence appears "apple green" in color.
  - i. Pure Culture Bacterial Confirmatory Testing: Positive bacterial isolates will fluoresce strongly and have the same morphology and size as the positive control. Negative isolates will not fluoresce.
  - ii. *R. salmoninarum* Screening: Examine at least 50 fields. Positive bacterial cells are 1.0 X 0.5 microns, which fluoresce. Negative smears will not fluoresce.

#### 5. Hints for Good Results:

- a. Use FITC conjugates at optimum working dilution. Follow manufacturer's recommendation to test for optimum working concentration (3.6.F).
- b. Filter all conjugated antibody reagents (.45um filter) prior to use to reduce background debris that fluoresce nonspecifically and cause difficulty in reading and interpreting the slides.
- c. Prepare thin smears; thick smears will not fix properly and are more easily washed off during the staining process, and thick slides require frequent focusing to observe multiple focal planes.

- d. Evenly distribute the kidney material in PBS (3.6.D.3), or use a very light inoculum of pure bacterial culture for each well (excess bacteria will stain poorly).
  - e. Heat-fix slides prior to fixing in acetone. If there is not possible to heat-fix the slides they can be air-dried and sent to the lab without fixation. Fixed slides should be stored refrigerated until stained.
  - f. Control slides should be rinsed in separate Coplin jars to avoid any potential for cross contamination during the staining process. Fixing solutions should be completely changed at a minimum between separate inspection cases, and/or when positive results occur.
  - g. Use anhydrous acetone to fix slides; the acetone reduces the lipid content of the preparation (de-fatting) increasing the overall fluorescence quality and intensity.
6. Commercial Sources for Antibodies:
- a. DiagXotics, Inc.  
 27 Cannon Rd., Wilton, CT 06897 USA  
 Phone: 800-676-2927, 203-762-0279  
 Web Site: <http://www.diagxotics.com>  
 Antibodies Available: Monoclonal antibodies available in FITC-conjugated and other preparations for the following bacterial pathogens; *Renibacterium salmoninarum*, *Aeromonas salmonicida*, *Edwardsiella ictaluri*.
  - b. Kirkegaard and Perry Laboratories, Inc.  
 2 Cessna Court, Gaithersburg, MD 20879-4145 USA  
 PHONE: 800-638-3167, 301-948-7755  
 Web Site: <http://www.kpl.com>  
 Antibodies available: Polyclonal antibodies available in FITC-conjugated and other preparations for *Renibacterium salmoninarum* only. KPL also provides positive control material for FAT.
  - c. Microtek International, LTD (Bayotek)  
 6761 Kirkpatrick Crescent, Saanichton, B.C., CA  
 Phone: 250-652-4482  
 Web Site: <http://www.microtek-intl.com>  
 Antibodies available: Polyclonal antibodies available for Indirect FAT for the following bacterial pathogens; ; *Renibacterium salmoninarum*, *Aeromonas salmonicida*, *Yersinia ruckeri* serotypes 1 and 2

### **3.8 Glossary**

**Acute** – a disease having rapid onset, severe symptoms and a relatively short course – not chronic.

**Anneal** – the attachment of oligonucleotide primers to a specific site on a single stranded DNA segment.

**Aseptic** – the nature of preventing contamination of foreign microorganisms.

**Basal media** – a media formulation to which additional components may be added for a particular test.

**Carbohydrate** – a particular sugar used in a test.

**Chronic** – describes the course of a disease which is long and drawn out – not acute.

**Commercially prepared** – a component or test which is available from a commercial source for purchase.

**Confirmatory identification** – identification of a pathogen through the completion of both initial screening techniques, and another confirmatory test as described in this document, the results of which concur with each other for positive identification of a particular organism.

**Counterstain** – a stain used in FAT to achieve a dark background color of tissues and materials stained, aiding in the observation of fluorescing bacterial cells.

**Decolorize** – the application of a solution to stained material to remove excessive stain.

**Denature** – the enzymatic/temperature dependent activity which converts double stranded DNA to single stranded DNA.

**DNA extraction** – the process of obtaining pure, double stranded DNA from sample tissues and materials.

**Elution** – the washing of DNA from a spin column filter membrane.

**Extension** – the synthesis of a new, copied segment of DNA following denaturing and annealing processes involved with PCR.

**Fermentation** – bacterial utilization of a compound in the absence of oxygen.

**Filtrate** – the resultant liquid obtained from filtration

**FITC** – fluorescein isothiocyanate, a reagent which is used as an antibody label for the fluorescent antibody test.

**FITC-conjugated (antibody)** – describes the existence of a fluorescent label on an antibody used for the fluorescent antibody test.

**Lysate** – the product solution of tissue lysis.

**Nested-PCR** – a second PCR is performed, targeting a sub-segment of DNA produced in the first round PCR specific for a particular organism.

**Oxidation** – bacterial utilization of a compound in the presence of oxygen.

**Pellet** – the product of sedimentation of solid materials from a liquid resulting from centrifugation.

**Phenotypic characterization** – the detectable expression of a bacterial isolate to environmental conditions, biochemical testing and morphological observations recorded for the purpose of differentiation and determination of the species of bacteria.

**Polymerase chain reaction (PCR)** – a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA.

**Positive control isolates** – bacterial isolates may be obtained from the American Type Culture Collection (ATCC: <http://www.atcc.org>).

**Post-dwell** – the period of time after cycles are completed in PCR.

**Presumptive identifications** – identification of a pathogen through the completion of initial screening tests as described in this document, where no other test has been performed to confirm the positive identification of the organism.

**Primary Culture** – bacterial cultures achieved from media inoculated directly from fish tissues.

**Primer** – oligonucleotides that, in the presence of DNA and excess dNTP's, hybridize specifically to a target sequence and “prime” new DNA synthesis.

**Pure Bacterial Culture** – a culture of bacteria originating from an isolated colony.

**Quality Control** – Taking steps to assure that testing and results are accurate and reliable.

**Selective media** – a medium containing ingredients which may either exclude growth of some microorganisms, select for growth of a particular species of microorganism, or both.

**Serological methods** – detection methods which employ the use of an antibody against the target organism.

**Spin column** – a small filter unit provided by many commercial DNA extraction kits used to

bind, wash and elute DNA from tissue lysates.

**Stock suspension** – suspension of a compound which must be further diluted before direct application in a particular test.

**Sub-acute** – a course of disease which exhibits some acute and some chronic symptoms.

**Subculture** – the transfer of an established bacterial culture to an uninoculated medium.

**Supernatant** – the surface fluid resulting from centrifugation of a liquid.

**Working suspension (dilution)** – suspension of a compound at the correct dilution for direct application in a particular test.

**Yellow-pigmented bacteria** – bacterial isolates which exhibit a yellow pigmentation on colony formations (e.g. *Flavobacterium* species).

### **3.9 References**

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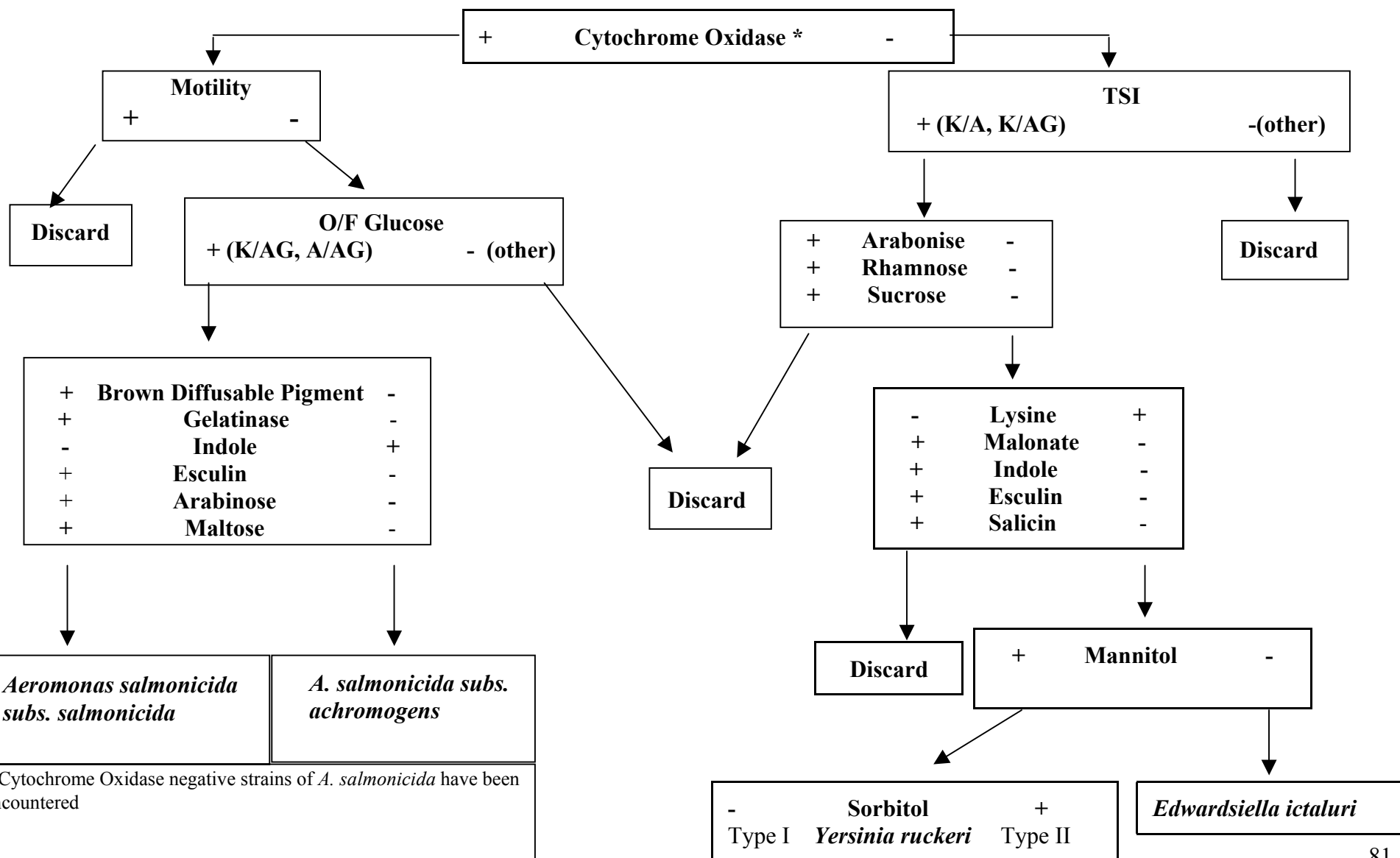
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**3.A1 - Laboratory Reference Flow Chart for identification of Gram Negative Bacterial Pathogens which grow on TSA or BHIA and are targeted for detection to complete Fish Health Inspection requirements.**



### **3.A2 - Profiles Obtained with API-20E for Known Fish Pathogens.**

The following table represents API20E profiles for *Yersinia ruckeri* when cultures were tested at 22°C rather than the manufacturer's recommended incubation temperature of 35-37°C. All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT. Based on the profile submitted to API, bacterial identification is given in order of probability, then remarks as to the likelihood of the profile are provided when profiles are poorly matched to the manufacturer's database (National Wild Fish Health Survey Lab Procedures Manual, USFWS).

<b>Bacterial Isolate ID</b>	<b>API PROFILE*</b>	<b>API Manual or Computer Identification</b>
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #1- Type II (11.29) 2. Nisqually Fall chinook (3/88)	<b>5307500</b>	1.) <i>Serratia marcescens</i> 2.) <i>Serratia liquefaciens</i> 3.) <i>Hafnia alvei</i>
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #2- Type II (11.29) 2. Fall Chinook, Suquamish R, WA (3/88) 3. Spring Chinook, Skookum Creek, WA (2/88)	<b>5107500</b>	(Same ID as 5307500 above) 1.) <i>Serratia marcescens</i> 2.) <i>Serratia liquefaciens</i> 3.) <i>Hafnia alvei</i>
Unknown source – Isolate confirmed by biochemical and serological testing.	<b>5144100</b>	1.) <i>Escherichia coli</i> 2.) <i>Yersinia ruckeri</i>
Eastern Fishery Disease Laboratory (EFDL) Positive Control - Type I (11.4)	<b>5107100</b>	"Unacceptable profile"
Coho, Quinault River, WA	<b>5106100</b>	"Questionable ID"
Late Fall Chinook, Battle Creek, CA (11/94)	<b>5105100</b>	1.) <i>Hafnia alvei</i> "Acceptable ID"
Notes from ERM archived files – previous testing	<b>5104500</b>	"Questionable ID"

### **3.A2 (continued)**

<b>Bacterial Isolate ID</b>	<b>API PROFILE*</b>	<b>API Manual or Computer Identification</b>
1. Hagerman – Type I (11.4)	<b>5104100</b>	1.) <i>Yersinia ruckeri</i> “Very good ID”
Coho, Quilcene R., WA (11/88)	<b>5104000</b>	1.) <i>Yersinia ruckeri</i> “Very good ID”
Unknown source	<b>5100100</b>	1.) <i>Yersinia ruckeri</i> “Excellent ID”
Unknown source	<b>4105100</b>	1.) <i>Hafnia alvei</i>
Unknown source	<b>4104100</b>	1) <i>Yersinia ruckeri</i> 2) <i>Salmonella gallinarum</i>
Unknown source	<b>4104000</b>	1.) <i>Yersinia ruckeri</i> “Acceptable ID”
Unknown source	<b>0104100</b>	1.) <i>Yersinia ruckeri</i> “Acceptable ID”

\* *Yersinia ruckeri* generally fails to produce a positive citrate reaction when incubated at room temperature (22-25°C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles. Also see references listed on page 5-27.

### **3.A2 (continued)**

The following represents API20E profiles for *Aeromonas salmonicida* isolates following manufacturer's instructions but incubating test strips at room temperature (22°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

<b>Bacterial Isolate ID (collection date)</b>	<b>API PROFILE*</b>	<b>Computer/Manual ID</b>
spp not identified, Makah NFH, WA (8/88)	<b>0006104</b>	<i>Pseudomonas pseudomaleae</i> "Acceptable ID"
Winter Steelhead, Makah NFH, WA (1/89) Chum, Makah NFH, WA (12/89)	0006104	Same as above
Winter Steelhead, Quinault NFH, WA (1/89)	0006104	Same as above
Spring Chinook, Entiat NFH, WA (8/89)	0006104	Same as above
Spring Chinook, Quilcene NFH, WA (3/91)	<b>2006104</b>	<i>Aeromonas salmonicida</i>
Profiles given in <b>API MANUAL</b> for <i>Aeromonas salmonicida</i>	<b>6006104</b> <b>6006504</b> <b>4006104</b> <b>2006104</b>	<i>Aeromonas salmonicida</i> "Good to Excellent ID"

\**Aeromonas salmonicida* generally fails to produce positive relations for ONPG, ADH, and LDC when incubated at room temperature (22-25° C).

Profiles provided in the API Manual are based on positive reactions for some or all of these first 3 biochemical tests, therefore the first digit of the "acceptable" profiles for *A. salmonicida* include the values 2,4, or 6. More often, a zero value is obtained after 24-48 hours incubation at room temperature. Longer incubation periods are required for these tests.

#### **References:**

Romalde, J.L., and A.E. Toranzo. 1991. Evaluation of the API-20E system for the routine identification of the enteric redmouth disease. Bull. Eur. Ass. Pathol. 11(4), 147.

Kent, M.L. 1982. Characteristics and identification of *Pasteurella* and *Vibrio* species pathogenic to fishes using API-20E (Analytab Products) multitube test strips. Can. J. Fish. Aquat. Sci.,

39. 1725-1729.

Toranzo, A.E., Y.Santos, T.P. Nieto and J.L. Barja. 1986. Evaluation of different assay systems for identification of environmental *Aeromonas* strains. *Appl. Environ. Microbiol.*, 51:652-656.

### **3.A3 PCR Worksheets**

#### **A. Worksheet A - PCR Sample Data/Log Sheet**

### PCR Sample Data/Log Sheet

Case Number \_\_\_\_\_ Sample Site \_\_\_\_\_ Date \_\_\_\_\_

Species \_\_\_\_\_

Tissue type \_\_\_\_\_

Tissue Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			

**B. Worksheet B - Amplification of Nucleic Acid by PCR for the Confirmation  
of *Renibacterium salmoninarum***



### **WORKSHEET 3.A3.B: Initial Amplification of *R.salmoninarum* DNA by PCR.**

**Case Number** \_\_\_\_\_

**Date** \_\_\_\_\_

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (μL) (to total 50μl)	Volume for _____ samples
d-H <sub>2</sub> O*		Add to total 40μL		30.1	
10XBuffer		1X	10X	5.0	
MgCL <sub>2</sub>		1.5mM	50mM	1.5	
dNTP's		0.2mM	10mM	1	
(+)Primer		20pMole	20pMole/μl	1	
(-)Primer		20pMole	20pMole/μl	1	
TAQ		2 units/Rx	5U/μl	0.4	
DNA <sup>±</sup>		-	-	10 μl	-

\*Add water to Master Mix first, TAQ last.

\*\*Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations.

<sup>±</sup>Do not add DNA template until Master Mix reaction tubes have been removed from the reagent Mixing (MM) area.

#### **Primer Sets for *R. salmoninarum* 1<sup>st</sup> Round Amplification**

<b>P3 (round 1 forward)</b>	5'-A GCT TCG CAA GGT GAA GGG-3'
<b>M21 (round 1 reverse)</b>	5'-GC AAC AGG TTT ATT TGC CGG G-3'

**C. Worksheet C - Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum***

**WORKSHEET 3.A3.C: Nested Amplification of *R.salmoninarum* DNA by PCR.**

Case Number \_\_\_\_\_

Date \_\_\_\_\_

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50µl)	Volume for ____ samples
d-H <sub>2</sub> O*		Add to total 49µL		39.1	
10XBuffer		1X	10X	5.0	
MgCL <sub>2</sub>		1.5mM	50mM	1.5	
dNTP's		0.4mM	10mM	1	
(+)Primer		20pMole	20pMole/µl	1	
(-)Primer		20pMole	20pMole/µl	1	
TAQ		2 units/Rx	5U/µl	0.4	
DNA <sup>±</sup> (round 1)		-	-	1 µl	-

\*Add water to Master Mix first, TAQ last. \*\*Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. <sup>±</sup>Do not add DNA template until Master Mix reaction tubes have been removed from the reagent Mixing (MM) area.

**Primer Sets for *R. salmoninarum* 2<sup>nd</sup> (Nested) Round Amplification**

<b>P4 (round 2 forward)</b>	5'-AT TCT TCC ACT TCA ACA GTA CAA GG-3'
<b>M38 (round 2 reverse)</b>	5'-C ATT ATC GTT ACA CCC GAA ACC-3'

Gel Concentration		Weight of agarose (grams)	Volume of Buffer (ml)

### Gel Template (Sample Placement Map)

Ladder Brand / Lot # \_\_\_\_\_ Loading Buffer Brand / Lot # \_\_\_\_\_

**Enter Sample ID below corresponding well number:**

PCR Products (Loaded LEFT to RIGHT)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

## **D. Worksheet D - Photodocumentation of the PCR Product Gel**

Case Number \_\_\_\_\_

Date:

Samples

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes: